



Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 13, pp. 1733-1738, 1997  
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 0960-894X/97 \$17.00 + 0.00

PII: S0960-894X(97)00300-4

## POTENT SELECTIVE THIENOXAZINONE INHIBITORS OF HERPES PROTEASES

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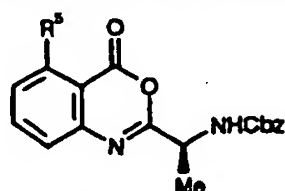
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**Abstract:** Thieno[3,2-d]oxazinones are potent, selective, mechanism-based inhibitors of the herpes proteases with good aqueous stability. Specificity between the HSV and CMV proteases varies across the series: compounds 14b and 14c are submicromolar HSV protease inhibitors with modest CMV protease inhibition, 14g is a selective CMV protease inhibitor, and 32 inhibits both enzymes with an IC<sub>50</sub> of about 1 µM.

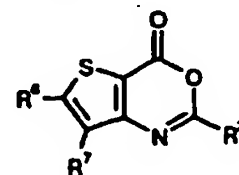
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The recent discovery that a protease is encoded by the UL26 gene of herpes simplex type 1 (HSV-1)<sup>1</sup> and by the homologous UL80 gene of cytomegalovirus (CMV),<sup>2</sup> has afforded a potential new target for therapy of herpesvirus infections. This protease plays an essential role in virus capsid maturation, cleaving a scaffold protein which is encoded in-frame with the C-terminal part of the gene.<sup>3</sup> The full-length protease also undergoes self-cleavage at two sites, the C-terminal maturation site (M site) which it shares with the scaffold protein, and the release site (R site) which results in release of the N-terminal catalytic domain. The protease shows a varying degree of sequence homology across the herpesvirus family and a highly conserved P4-P1' cleavage motif in which proteolysis occurs between alanine and serine residues. The herpes proteases do not show homology with any known proteases and the recent determination of the crystal structure of CMV protease indicates that they belong to an entirely new family of serine proteases with a novel catalytic Ser-His-His catalytic triad.<sup>4</sup>

We recently reported the first potent mechanism-based inhibitors of the herpes proteases.<sup>5,6</sup> These inhibitors included the known serine protease inhibitor class of 2-substituted benzoxazinones such as 1,<sup>5</sup> and spiro-oxazolones and imidazolones which represented novel serine protease inhibitor classes.<sup>6</sup>



1a R<sub>5</sub> = Me  
 1b R<sub>5</sub> = H



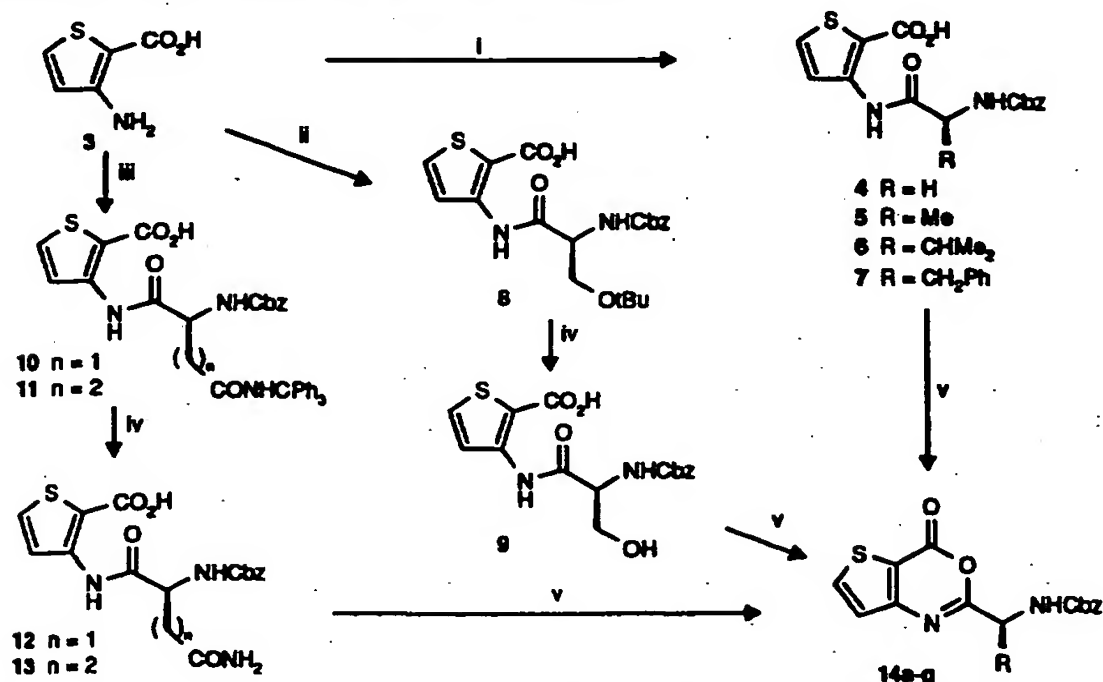
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It was noted that for a series of 5-substituted benzoxazinones with a 2-substituent derived from Cbz-alanine, the HSV-1 protease inhibitory potency was inversely dependent on the size of the 5-substituent. Thus in the general structure 1 compounds with R<sup>5</sup> as chloro or ethyl were not inhibitory, whereas compound 1a with R<sup>5</sup> as methyl had low potency and 1b with R<sup>5</sup> as hydrogen was moderately potent. In order to reduce the

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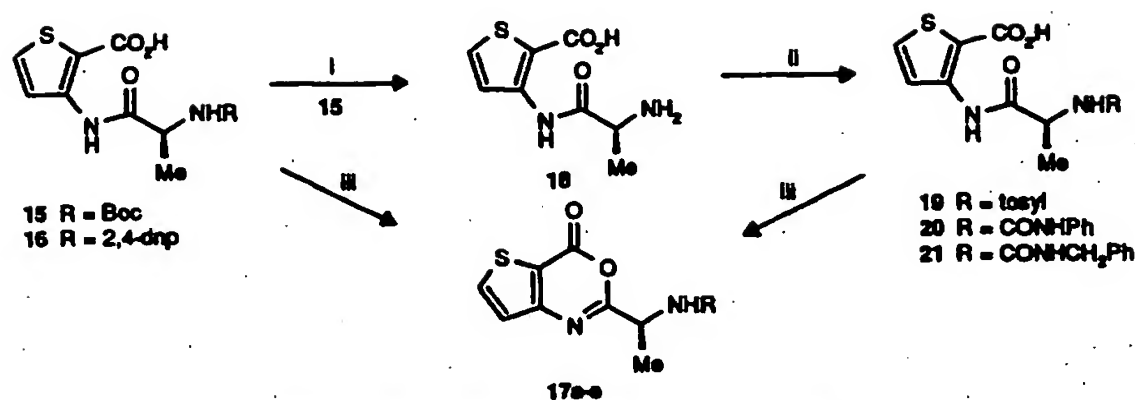
steric bulk at this position still further, we decided to prepare a series of derivatives of the 4*H*-thieno[3,2-*d*][1,3]oxazin-4-one ring system 2.

Synthesis of 2-substituted thienoxazinones with different branching groups was achieved by acylation of 3-aminothiophene-2-carboxylic acid 3 with a variety of amino acid derivatives using the mixed anhydride from *iso*-butyl chloroformate (Scheme 1 and Table 1). Non-functionalised amino acid derivatives 4-7 were then cyclised with water soluble carbodiimide, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (DEC). Attempted one-step acylation/cyclisation using DEC resulted in decarboxylation of the thiophene ring. In the case of serine the *O*-*t*-butyl protecting group was used and this was removed at the intermediate stage 8 with trifluoroacetic acid (TFA). For the asparagine and glutamine side chains, trityl protection was used and this was again removed with TFA at the intermediate stage (10 and 11).



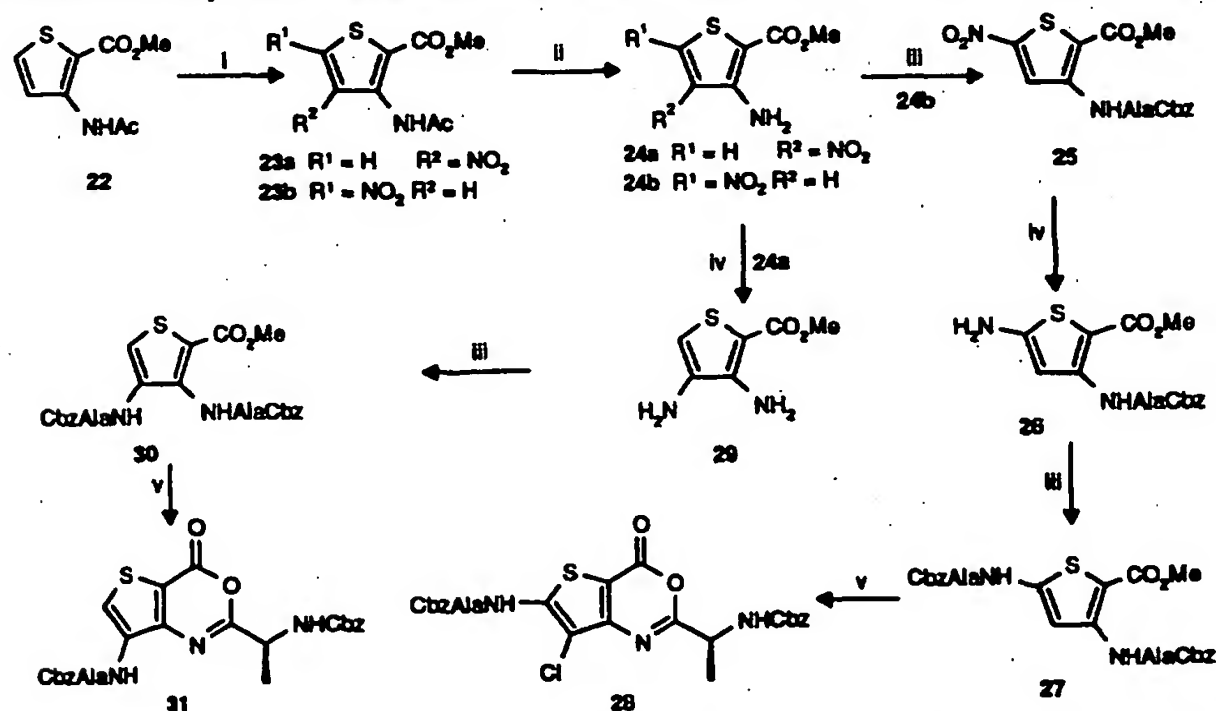
Scheme 1. i. *i*BuOCOC/*N*-methylmorpholine(NMM)/DMF/Cbz-Gly, Cbz-Ala, Cbz-Val or Cbz-Phe; ii. *i*BuOCOC/NMM/DMF/Cbz-Ser(*t*Bu); iii. *i*BuOCOC/NMM/DMF/Cbz-Asn(trityl) or Cbz-Gln(trityl); iv TFA/CH<sub>2</sub>Cl<sub>2</sub>; v. DEC/DMF.

Analogue of 14b in which the Cbz group was replaced by Boc or 2,4-dinitrophenyl were prepared by acylation of the aminothiophene 3 with the appropriately protected alanine affording the intermediates 15 and 16 which were cyclised by DEC treatment to afford 17a and 17b (Scheme 2 and Table 1). Deprotection of 15 yielded the amine 18 as the hydrochloride in 55% yield. Reaction of 18 with tosyl chloride or the appropriate isocyanate afforded 19-21 which were cyclised in the usual way to 17c-e. In the case of tosyl chloride some spontaneous cyclisation occurred in the tosylation reaction.



Scheme 2. i. 6M HCl/dioxane/anisole; ii. TsCl/*i*Pr<sub>2</sub>EtN/DMF or RNCO/pyridine/60°C; iii. DEC/DMF.

The 6-phenyl and 7-methyl derivatives of 14b were prepared in the usual way from the relevant substituted thiophene amino acid. From our studies of benzoxazinone inhibitors,<sup>5</sup> aminoacyl substituents in the thiophene ring were of interest and the synthesis of these compounds commenced with nitration of the 3-aminothiophene ester 22. When the nitration of 22 was carried out between -30 and -25°C,<sup>7</sup> the 5-nitro isomer 23b was obtained exclusively in an isolated yield of 38%. However, nitration of 22 under conditions similar to those described by Elliott *et al.*,<sup>8</sup> afforded a mixture of 4- and 5-nitrothiophenes 23a and 23b in a ratio 1:1.4 which was separated by chromatography on silica gel. Since literature reports regarding the assignment of the site of nitration of thiophene 22 are inconsistent,<sup>7-9</sup> we decided to determine unequivocally the regiochemistry of isomers 23a and 23b. Initial analysis of the NMR chemical shifts and 2D heteronuclear correlations were not conclusive and definitive evidence for assignment of the regioisomers was acquired using secondary isotope shifts observed after partial NH deuteration.<sup>10</sup> The acetyl groups in 23a and 23b were removed to give the



Scheme 3. i. H<sub>2</sub>SO<sub>4</sub>/HNO<sub>3</sub>; ii. HCl/MeOH; iii. Cbz-Ala/BuOCOCiNMM; iv. Fe/AcOH; v. PCl<sub>5</sub>/CCl<sub>4</sub>/CHCl<sub>3</sub>.

aminothiophenes 24a and 24b.<sup>8</sup> Acylation of the amino function in 24b with the mixed anhydride of Cbz-Ala provided compound 25 which was reduced to the 5-aminothiophene 26 and this in turn was acylated to afford diamide 27. The isomeric diamide 30 was prepared similarly from 24a but in this case reduction to 29 preceded diacylation. Cyclisation of 27 and 30 to the oxazinones 28 and 31 was effected with  $\text{PCl}_5/\text{CCl}_4$ <sup>11</sup> and proceeded with concomitant chlorination of the thiophene ring in the case of 27.

The thiophene oxazinones were evaluated in hplc assays of the peptidolytic activity of the HSV-1, HSV-2 and CMV proteases.<sup>12</sup> The Cbz-alanine derivative 14b was found to be a submicromolar inhibitor of HSV-1 protease (Table 1) with a 100-fold improvement on potency over the benzoxazinone analogue 1b. The serine derived compound 14c was also a potent HSV-1 protease inhibitor. Both compounds inhibited HSV-2 protease at similar levels to HSV-1 protease, as might be expected from the high sequence homology of the two proteins. The nature of the  $\text{R}^1$  group (Table 1) had a profound effect on inhibitory potency. For optimal HSV-1 protease inhibition, small  $\text{R}^1$  groups are preferred and potency is reduced with larger groups to the point where the phenylalanine 14g is completely ineffective. In contrast, compounds with small  $\text{R}^1$  groups were much less effective inhibitors of CMV protease and in this case compound 14g was optimal.

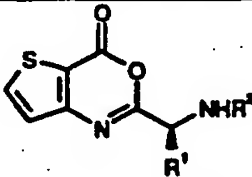
					
No.	$\text{R}^1$	$\text{R}^2$	$\text{IC}_{50}$ ( $\mu\text{M}$ ) or inhibition at 10 $\mu\text{M}$		
			HSV-1	HSV-2	CMV
14a	H	Cbz	2.7	ND	45
14b	Me	Cbz	0.48	0.42	9.4
14c	$\text{CH}_2\text{OH}$	Cbz	0.65	0.33	20
14d	$\text{CH}_2\text{CONH}_2$	Cbz	10	ND	18
14e	$(\text{CH}_2)_2\text{CONH}_2$	Cbz	6.3	ND	ND
14f	$\text{CHMe}_2$	Cbz	31	ND	26
14g	$\text{CH}_2\text{Ph}$	Cbz	>300	ND	3.5
17a	Me	Boc	ND	32%	15%
17b	Me	2,4-dinitrophenyl	ND	2.9	3.3
17c	Me	tosyl	ND	13%	30%
17d	Me	$\text{CONHPh}$	ND	>100	>100
17e	Me	$\text{CONHCH}_2\text{Ph}$	ND	1%	20%

Table 1. Inhibition of herpes proteases by 2-substituted thieno[3,2-d]oxazinones.

Replacement of the Cbz group with Boc, sulphonamide or urea substituents (17a, c-e) resulted in a significant decrease in HSV-2 protease potency but the dinitrophenyl derivative 17b afforded increased CMV inhibition, a similar  $\text{IC}_{50}$  being obtained for both HSV-2 and CMV enzymes.

Our previous studies had shown that a Cbz-alanine amide substituent on the 7-position of the aryl ring of benzoxazinones increased HSV-1 protease inhibition.<sup>5</sup> In thiophene oxazinones the Cbz-Ala seems to be

preferred at the 6-position (32, Table 2). This substituent notably enhanced CMV protease potency so that 32 had a similar effect on the HSV-2 and CMV enzymes. In contrast, substitution at this position with a phenyl ring significantly reduced CMV potency (compound 30).

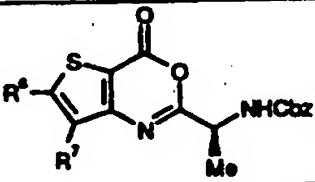
					
No.	R <sup>6</sup>	R <sup>7</sup>	IC <sub>50</sub> (μM) or inhibition at 10 μM		
			HSV-1	HSV-2	CMV
30	Ph	H	3.3	ND	>300
31	H	Me	3.1	ND	12
32	Cbz-Ala-NH	Cl	ND	1.6	1.3
33	H	Cbz-Ala-NH	ND	50%	43%

Table 2. Inhibition of herpes proteases by ring substituted thieno[3,2-d]thioxazinones.

The SAR of these thieno[3,2-d]oxazinones for HSV and CMV proteases clearly do not run in parallel. Compound 30 has about 100-fold selectivity for HSV-1 over CMV protease whereas compound 14g has similar selectivity in the opposite direction. Despite their similar cleavage pattern, there is significant sequence divergence between the HSV and CMV enzymes (30% identity). However, compounds such as 32 do have micromolar potency for both HSV and CMV proteases, suggesting pan-herpetic inhibition may be achievable.

In order to check that protease inhibition was occurring by formation of a covalent adduct as anticipated, compound 14b was incubated with HSV-2 protease and adduct formation was monitored by electrospray mass spectroscopy. At both 2 min and 60 min time-points a monoadduct with mass increment of 330 was observed, consistent with the thioxazinone acting as a mechanism-based inhibitor of HSV-2 protease by formation of an acyl-enzyme complex.

The selectivity of 14b with respect to representatives of the major serine protease families, elastase and subtilisin was examined. Virtually no inhibition of subtilisin was observed up to an inhibitor concentration of 1 mM. Compound 14b was also very selective with respect to elastase, with an IC<sub>50</sub> of 120 μM. Although the absolute value of the selectivity ratio depends on the assay conditions, it is interesting that decreasing the steric bulk in the region peri to the oxazinone carbonyl ring from compound 1b through to 14b results in a 10<sup>5</sup>-fold increase in the relative selectivity ratio (Table 3). Analysis of the aqueous stability of 14b as described previously<sup>5</sup> also indicated that it had enhanced stability relative to the benzoxazinones 1a and 1b (Table 3).

Compound	IC <sub>50</sub> (μM)		elast/HSV-1 pr.	t <sub>1/2</sub> (h)
	HSV-1 pr.	elastase		
1a	120	0.22	0.002	33
1b	50	45	1.1	14
14b	0.48	120	250	>100

Table 3. Comparison of 14b with 5-substituted benzoxazinone analogues 1a and 1b.

Replacement of the benzene ring of 2-carba benzoxazinones with a thiophene ring thus resulted in thieno[2,3-d]oxazinone inhibitors such as 14b, 14c and 32 which are potent and selective mechanism based inhibitors of the herpes proteases with enhanced stability relative to benzoxazinones.

**Acknowledgements** We thank Dr D.G. Tew and Dr J.E. Strickler for the elastase and subtilisin assays, Dr C.J. Salter and Mrs C.A. Reid for stability determinations and Dr S.A. Readshaw for advice on the NMR methods.

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10. The magnitude of the  $^1\text{J}_{\text{CH}}$  heteronuclear coupling constants for the protonated carbons of compounds 23a and 23b (194.2 Hz and 186.2 Hz respectively in  $\text{CDCl}_3$ ) suggested that 23a possessed a C5 protonated carbon and 23b possessed a C4 protonated carbon although the difference in  $^1\text{J}_{\text{CH}}$  values for the two compounds was smaller than expected from literature data for thiophenes.<sup>9b</sup> Secondary isotope shifts,  $^n\Delta\delta$  where  $\Delta\delta = \delta\text{C(D)} - \delta\text{C(H)}$  and  $n$  = the number of intervening bonds, were determined in  $d_6$ -acetone. For 23b, large  $^2\Delta\delta$  effects were observed for C3 (-134 ppb) and 3-NHCO (-97 ppb) and smaller  $^3\Delta\delta$  effects for C2 (-38 ppb) and the protonated carbon (-52 ppb). On this basis the protonated carbon for 23b was assigned as C4 with the position of nitro substitution therefore at C5. Based on this conclusion, together with corroborating  $^1\text{J}_{\text{CH}}$  values, thiophene 23a must possess a C5 protonated carbon with nitro-substitution at C4. The observed  $^n\Delta\delta$  for 23a were consistent with this assignment, where  $^n\Delta\delta$  of C3, 3-NHCO, C2 and 3-NHCOMe were of a similar magnitude for 23a as for 23b but no  $^n\Delta\delta$  was observed to either the protonated or nitrated carbon of 23a. The lack of the expected  $^n\Delta\delta$  for C4 of 23a could be explained by the extensive quadrupolar broadening observed for the nitrated carbon of both 23a and 23b arising from the adjacent nitrogen. The  $^n\Delta\delta$  values are consistent with literature data for indoles and pyrroles<sup>13</sup> and the conclusions corroborate those drawn from  $^1\text{J}_{\text{CH}}$  values above and in reference 8.
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(Received in Belgium 3 April 1997; accepted 5 June 1997)